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NOVEL THERAPY FOR NEUROBLASTOMA; FOCUS ON PROSTAGLANDIN E2 AND MICROSOMAL PROSTAGLANDIN E SYNTHASE-1

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Novel Therapy for Neuroblastoma; Focus on Prostaglandin E2 and Microsomal Prostaglandin E synthase-1

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ABSTRACT

Cancer is a major public health problem and to date cancer is one of the leading causes of death worldwide. Neuroblastoma is a malignant pediatric tumor of the sympathetic nervous system. Neuroblastoma is one of the most common and deadliest extra cranial tumors of childhood. During the last decades, the survival rate has increased for children with neuroblastoma. However, this mainly accounts for children with favorable tumor biology while only 40-50% of children with high-risk neuroblastoma survive their disease despite very intensive treatment regimen. This demands a better understanding of high-risk neuroblastoma biology to enable novel therapeutic approaches.

Solid tumors are composed by a variety of cellular components including malignant cells and non-transformed stromal cells. The complex interaction between these cells creates the tumor microenvironment and contributes to tumor growth and initiation. Prostaglandin E_2 (PGE_2) regulates tumor inflammation and immune suppression, angiogenesis, metastasis, tumor progression and therapy resistance.

PGE_2 production is conducted via the conversion of arachidonic acid (AA) by the cyclooxygenase enzymes (COX-1 and COX-2) into the intermediate prostaglandin H_2 (PGH_2), subsequently converted by microsomal prostaglandin E synthase-1 (mPGES-1), the terminal enzyme responsible for induced PGE_2 formation. Inhibitors of the COX enzymes are potent preventive agents in several malignancies. However, clinical use of COX inhibitors in oncology has been hampered due to side effects caused by unselective inhibition of all products downstream of PGH_2 that are important for normal cellular functions. In this thesis we improve the biological understanding of PGE_2 in neuroblastoma and investigate if inhibition of mPGES-1 in neuroblastoma could maintain the anti-carcinogenic effect of COX targeting without the severe side effects.

Investigation of neuroblastoma tissues and cell lines revealed an abundant expression of all PGE_2 receptors and PGE_2 increased neuroblastoma cell growth and induced activation of survival signaling cascades. Inhibition of PGE_2 receptor signaling reduced cell survival. Investigation of aggressive high-risk neuroblastoma subsets revealed a highly activated PGE_2 synthesis pathway with elevated levels of PGE_2 in tumors due to high mPGES-1 expression and low expression of 15-PGDH, responsible of PGE_2 degradation. Compared to adult malignancies little is known of the role of inflammation in childhood cancers. Analysis of the microenvironment of the high-risk tumors showed a higher infiltration of immunosuppressive macrophages compared to low-risk tumors indicating a tumor-promoting inflammatory local milieu. Furthermore, we could show that infiltrating cancer-associated fibroblasts (CAFs) in the neuroblastoma microenvironment was the major source of mPGES-1 expression. Pharmacological inhibition of mPGES-1 in preclinical *in vivo* models modulated the microenvironment towards a less tumor-promoting state and significantly reduced tumor growth. In an established *in vitro* model recapitulating *in vivo* features of neuroblastoma, inhibition of mPGES-1 augmented the cytotoxic effect of conventional chemotherapeutics.

In this thesis it is concluded that mPGES-1 targeting represents a promising novel therapy in neuroblastoma. Further, the importance of deepened understanding of the complex interactions and heterogeneity within the neuroblastoma microenvironment is underlined.

LIST OF SCIENTIFIC PAPERS

I. Rasmuson A, **Kock A**, Fuskevåg OM , Kruspig B, Simon- SantaMaria J, Gogvadze V, Johnsen IJ, Kogner P*, Sveinbjörnsson B*. Autocrine Prostaglandin E2 Signaling Promotes Tumor Cell Survival and Proliferation in Childhood Neuroblastoma. PLoS ONE 2012 ; 7(1): 29331

II. Larsson K*, **Kock A***, Idborg H, Arsenian Henriksson M, Martinsson T, Johnsen JI, Korotkova M, Kogner P*, Jakobsson PJ*. COX/mPGES-1/PGE2 pathway depicts an inflammatory- dependent high-risk neuroblastoma subset. PNAS. 2015;112 (26):8070-8075

III. **Kock A**, Larsson K, Bergqvist F, Eissler N, Elfman L, Raouf J, Korotkova M, Johnsen JI, Jakobsson PJ*, Kogner P*. Selective inhibition of mPGES-1 in cancer-associated fibroblasts suppresses neuroblastoma tumor growth. Manuscript.

IV. **Kock A**, Korotkova M, Johnsen JI, Jakobsson PJ, Larsson K*, Kogner P* Establishing an in vitro model mimicking neuroblastoma microenvironment - opening up for combinational drug screening targeting cells of the stroma. Manuscript.

*Shared authorship

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALK	Anaplastic lymphoma kinase
APC	Antigen presenting cells
CAF	Cancer associated fibroblast
cAMP	cyclic AMP
COX	Cyclooxygenase
Coxibs	COX-2 inhibitors
cPGES	Cytosolic prostaglandin E synthase
PLA2	Phospholipase A2
CTLs	Cytotoxic T lymphocyte
ECM	Extracellular matrix
FAP	Fibroblast activating protein
FSP-1	Fibroblast specific protein
GD2	Disialoganglioside 2
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G-protein coupled receptors
IL	Interleukin
INFγ	Interferon γ
LPS	Lipopolysaccharide
MCTS	Multicellular tumor spheroids
mPGES	Microsomal prostaglandin E synthase
MYCN	Neuroblastoma *MYC* oncogene
NF-κβ	Nuclear factor-kappa beta
NK-cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononucleated cells
PDGFR	Platelet-derived growth factor receptor
PGD_2	Prostaglandin D_2
PGDH	Hydroxyprostaglandin dehydrogenase

PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PHOX2B	Paired–like homeobox 2b
T reg	Regulatory T cells
TDTS	Tissue-derived tumor spheres
TGF-β	Transforming growth factor β
TNFα	Tumor necrosis factor α
TXA_2	Thromboxane
VGEF	Vascular endothelial growth factor
αSMA	α-smooth-muscle actin

1 INTRODUCTION

1.1 CANCER

Cancer is a group of diseases where cells of the body have transformed into abnormal cells that grow without control and have the ability to invade nearby tissues and spread to other parts of the body.

The development of cancer is thought of as a multi-step evolutionary process on the cellular level where cancer cells gain somatic gene mutations over time resulting in lost responses to normal signals that otherwise strictly controls cell growth and cell death. In the year 2000 Hanahan and Weinberg described six common traits that together is essential for cancer formation and progression; limitless replicative potential, self-sufficient in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, evading apoptosis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Besides proliferating cancer cells a tumor is composed of a diverse variety of stromal cells and the extracellular matrix, together constituting the tumor microenvironment. The non-malignant cells of the microenvironment function not only as a supportive structure but are actively contributing to cancer development and progression. Therefore, to fully understand tumorigenesis it is not enough to solely study the transformed cancer cells of the tumor (Bissell and Radisky, 2001; Hanahan and Weinberg, 2011; Kenny et al., 2007).

Childhood cancers and cancers that develop in adults differ. Adult cancers develop over a long period of time and can often be coupled to lifestyle or environmental exposure. The long latency for cancerous development in adults is due to the accumulation of mutations required to overcome the normal cellular controls (Knudson, 1971). On the contrary childhood cancers are due to defects in the normal development machinery and fewer genetic changes are needed for tumor development (Scotting et al., 2005).

To date cancer is a leading cause of death worldwide and is a major public health problem (Ferlay et al., 2015; Torre et al., 2015). The cancer incidence in Sweden is 60000 persons per year and it is constantly increasing(cancerfondsrapporten, 2017). Children diagnosed with cancer in Sweden is approximately 300 per year and cancer is the leading cause of death in children <15 years of age in Sweden (Barncancerfonden, 2017). The treatment regimens today for both adults and children are mainly based on surgery, irradiation and chemotherapy in different combinations. Today nearly 80% of children with cancer can be cured (Barncancerfonden, 2017). However, for the majority of childhood cancers the survival has almost been unchanged during the last 20 years. This suggest that the impact of the conventional treatments on survival have reached a plateau (Figure 1) (Gustafsson et al., 2013; Johnsen et al., 2009) In addition, radiation and chemotherapy are especially hazardous in children as they damage the normal developing organs. New innovative therapeutic approaches are therefore needed.

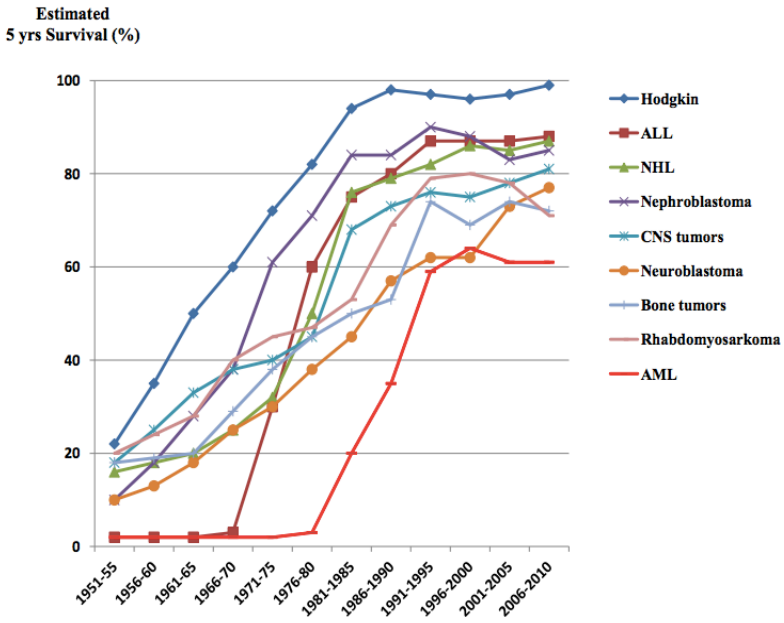

Figure 1. The estimated prognosis (5-years survival) over time for selected diagnostic groups. The prognosis improved considerably during time period 1970-1995. The results during the last decades seem to have reached a plateau. Reprinted from with permission from authors (Gustafsson et al., 2013).

1.2 NEUROBLASTOMA

Neuroblastoma is a malignant tumor of the sympathetic nervous system that mainly affects young children below one year of age. Neuroblastoma represents 7% of all childhood malignancies and accounts for 10% of cancer related deaths of young children (Johnsen et al., 2009; Park et al., 2014). This makes neuroblastoma the most common and deadliest extra cranial cancer in children. Neuroblastoma is a heterogeneous disease, with an extreme diversity in clinical presentation and prognosis ranging from spontaneous regression to metastatic aggressive tumors with poor prognosis (Brodeur, 2003; Matthay et al., 2016). During the last decade the survival of children with neuroblastoma has improved (Figure 1) (Gustafsson et al., 2013). However this mainly account for low-risk patients while patients with high-risk disease still have a poor prognosis with long term survival rates less than 50% in most populations despite intensive therapy (Maris, 2010; Park et al., 2014).

Figure 2. Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes and bone marrow, and in infants also spread to liver and subcutaneous tissue. Reprinted with permission of Springer Science media and Business Media. (Johnsen et al., 2009)

1.2.1 Biology and genetics

Neuroblastoma was first described as cancer of the developing, immature neural cells called neuroblasts in the 1800s by the German pathologist Rudolf Virchow and has since then fascinated clinicians and researchers due to its heterogeneous clinical presentations (Virchow, 1864). Neuroblastoma is thought to originate from neural crest cells, transient multipotent cells derived from the ectodermal germ layer that give rise to the peripheral nervous system (Knecht and Bronner-Fraser, 2002).The primary tumors are predominantly found in the adrenal medulla (65%) but can arise anywhere in the sympathetic nervous tissue along the spinal cord (Figure 2) (Brodeur, 2003; Cheung and Dyer, 2013; Maris, 2010). Some neuroblastomas have the extraordinary ability to spontaneously mature and regress. The mechanism of spontaneous regression is not fully understood but expression of TrkA, lack of TERT expression and host antitumor immunity has been suggested to have a role in tumor regression (Brodeur and Bagatell, 2014; Matthay et al., 2016).

Several genetic aberrations have been linked to neuroblastoma. The most common genetic alteration related to neuroblastoma includes neuroblastoma *MYC* (*MYCN*) oncogene amplification, and segmental chromosomal deviations such as 11q deletion, 17q gain and 1p deletion (Caren et al., 2010). The correlation of MYCN and advanced stages of neuroblastoma was discovered in the 1980s (Brodeur et al., 1984; Schwab et al., 1983). Amplification of *MYCN*, located on the short arm of chromosome 2 is one of the most frequent genetic deviation among high risk neuroblastomas, observed in approximately 30-40% of these patients and has routinely been used as biomarker for treatment stratification (Maris et al., 2007). Allelic loss of 1p36 is found in 20-30% of neuroblastomas and correlates with *MYCN*-amplification and poor outcome (Maris et al., 2007). Gain of 17q is the most common genetic alteration, found in more than 60% of all neuroblastomas and is often

detected in advanced tumors in children >1 year of age and is strongly associated with 1p-deletion and *MYCN*-amplification (Bown et al., 1999). Another common genetic alteration regularly found associated with poor prognosis in neuroblastoma is the segmental deletion of the long arm of chromosome 11 (11q-deletion). 11q-deletion commonly occurs in tumors with chromosome instability and are almost exclusively lacking *MYCN*-amplification and is therefore a useful predictive marker in unfavorable tumors without *MYCN*-amplification (Caren et al., 2010). Patients with 11q-deletion are often older at disease onset, 42 months compared to 21 months in children with *MYCN* amplified tumors, and have a slower disease progression but frequently develop therapy resistance. Patients with 11-q deleted tumors have similarly poor clinical outcome as the patients with *MYCN* amplified tumors (Figure 3) (Caren et al., 2010; Cetinkaya et al., 2013). It is clear that 11q-deletion and *MYCN*-amplification characterize two distinct groups of aggressive neuroblastoma and that a high frequency of 17q gain occurs in both of these groups. Neuroblastomas presented with only numerical chromosomal alterations show an excellent overall survival (Caren et al., 2010).

Hereditary neuroblastoma is rare and accounts only for 1-2% of all neuroblastoma cases. Paired–like homeobox 2b (PHOX2B) was the first predisposition mutation found in neuroblastoma, however only a small part of hereditary neuroblastoma could be explained by PHOX2B mutations. In 2008 it was reported that germ line mutations resulting in a constitutive activation of the anaplastic lymphoma kinase (ALK) was the main cause of familial neuroblastoma (Mosse et al., 2008). ALK-activating mutations was also found to be somatically acquired (Chen et al., 2008; George et al., 2008; Mosse et al., 2008). Both PHOX2B and ALK are involved in the development of the nervous system.

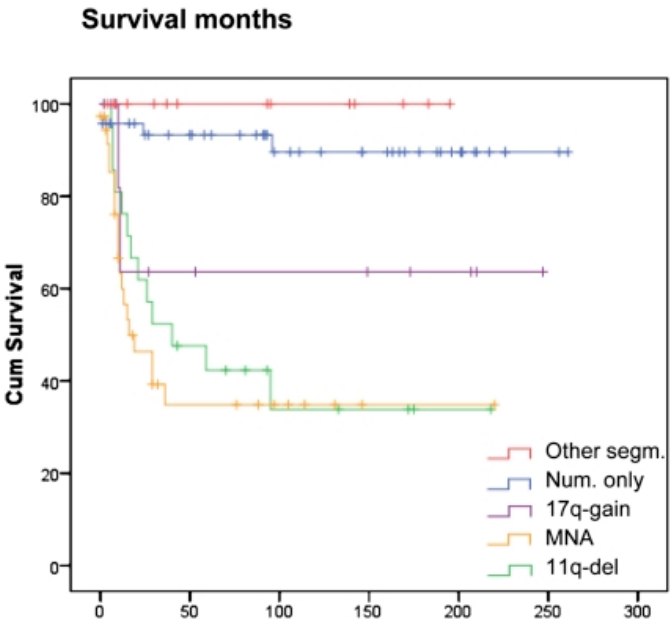

Figure 3. Kaplan-Meier overall survival for patients with tumors with different genomic profiles. The tumors are grouped as follows: the other segmental group (red line), the numerical-only group (blue line), the 17q-gain group (violet line), the MYCN-amplification group (yellow line), and the 11q- deletion group (green line). Reprinted with permission from Proceedings of the National Academy of Sciences of the United States of America. (Caren et al., 2010)

1.2.2 Staging, risk classification and treatment

The International Neuroblastoma Risk Group staging system (INGRSS) was developed as a new staging system in 2009 and is based on tumor imaging to stratify patients at the time of diagnosis before treatment instead of post-surgical evaluation. Centered on image-defined risk factors (IDRFs) loco regional tumors are staged as L1 or L2. Children with metastatic tumors are defined as stage M except for children below 18 months of age with metastatic spread to liver, skin and bone marrow that are defined as MS (Monclair et al., 2009). The stage of the disease is combined with other prognostic factors into a classification system that stratifies patients into pretreatment subgroups. The International Neuroblastoma Risk Group classification system defines neuroblastoma risk groups as very low, low, intermediate, and high based on histology, age at diagnosis and genetic aberrations such as MYCN status, 11q-deletion and DNA ploidy (Cohn et al., 2009).

Treatment of neuroblastoma is funded on which risk group the patients are stratified into. The treatment differ widely between low-risk and high-risk groups. Treatment of high-risk patients involves intense induction chemotherapy regimen including cisplatin, vincristine, carboplatin, etoposide and cyclophosphamide known as COJEC (Ladenstein et al., 2017; Pearson et al., 2008). The induction chemotherapy is then followed by surgery and myeloablative chemotherapy combined with reinfusion of hematopoietic stem cells and later local radiotherapy. Maintenance treatment consists of differentiating therapy with retinoic acid in combination with immunotherapy, including combinations of monoclonal antibodies targeting disialoganglioside 2 (GD2) and granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-2 (Ladenstein et al., 2010; Matthay et al., 2016; Park et al., 2014). Children in the intermediate-risk group receive a milder chemotherapy regimen followed by surgical resection of the remaining tumor. Treatment stratification in the low-risk groups aims to deliver minimum therapy, where some children can be cured solely by local surgery while no treatment may be needed for the metastatic neuroblastomas that spontaneously regress (Matthay et al., 2016). Patients with low- and intermediate-risk neuroblastoma have an overall survival rate above 90% while only 40-50% of the high-risk children will be long term survivors (Park et al., 2014).

1.3 THE TUMOR MICROENVIRONMENT

Solid tumors are not only composed of malignant cells. Solid tumors are heterogenic and composed of a variety of cellular components including cancer cells, endothelial cells, the non-cellular extracellular matrix (ECM), various immune cells and cancer-associated fibroblasts (CAFs) (Figure 4). The complex interplay between these components contributes to cancer initiation and promotes growth and metastasis. The “seed” and the “soil” hypothesis by Padget in 1898 was the first to describe the importance of the tumor microenvironment where the cancer cell (the seed) required a specific microenvironment (the soil) to establish and grow (Paget, 1989). As the tumor grow, cells in the microenvironment co-evolve and

modulates the cancer milieu into a state that actively contributes to cancer progression (Bissell and Radisky, 2001; Hanahan and Weinberg, 2011). This capability to change the surroundings is important for tumor cells to acquire some of the hallmarks of cancer. Thus targeting of the tumor microenvironment to destroy cancer cells in their local milieu has become an important tool in the overall cancer treatment (Martin et al., 2016). Different ways of targeting the stromal compartment within the microenvironment is frequently revealed and studies including anti-angiogenic drugs, immunotherapies that boost the immune responses against cancer, targeting-cancer associated fibroblast (CAF) markers and remodeling of the ECM have been initiated (Bonnans et al., 2014; Sharma et al., 2011; Sounni and Noel, 2013).

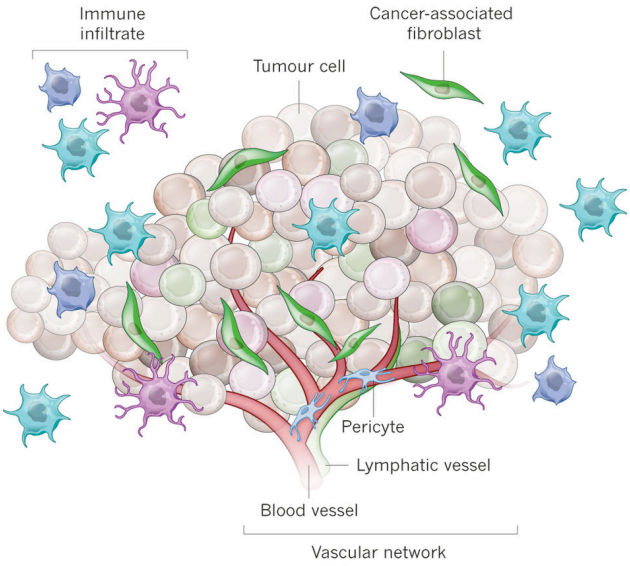

Figure 4. The tumor microenvironment. Tumor formation involves the co-evolution of neoplastic cells together with extracellular matrix and vascular endothelial, stromal and immune cells. Reprinted by permission from Macmillan Publishers Ltd: Nature, advance online publication, 19 September 2013 (doi:10.1038/sj.nature12626)

1.3.1 Cancer associated fibroblasts

Fibroblast is one of the most abundant cell type detected within the tumor microenvironment. Fibroblasts found in close proximity to cancer cells within the tumor microenvironment have been termed cancer-associated fibroblasts (CAFs). Fibroblasts are cells of the connective tissue that mediates wound healing by synthesizing the ECM, recruiting inflammatory cells and producing cytokines and chemokines. Cancer has been described as a wound that never heals implying that the processes associated with wound healing are comparable to those involved in the development and growth of tumor stroma (Dvorak, 1986). During wound healing fibroblasts are recruited and activated to repair damaged tissue, and when the healing process is completed activated fibroblasts decrease by undergoing apoptosis or by reverting to a quiescent state (Ohlund et al., 2014). In contrast, CAFs stays continuously activated.

Activated fibroblast within the tumor microenvironment display an altered phenotype and are characterized by expression of α-smooth-muscle actin (αSMA), fibroblast activating protein (FAP), Fibroblast specific protein (FSP-1), platelet-derived growth factor receptor α and β (PDGFR α and PDGFR β) and vimentin (Shiga et al., 2015). However, the heterogeneity in expression of these markers indicates that CAFs are polarized into distinct subpopulations

depending on their origin and the local tumor environment (Augsten, 2014; Sugimoto et al., 2006).

The origin of CAFs is still not totally clear. In general, CAFs have been considered to originate from local fibroblast stimulated by factors released by tumor cells. However CAFs have been reported to derive from several different cell types including mesenchymal stem cells, hematopoietic stem cells and from epithelial cells via epithelial to mesenchymal transition (Oumlhlund et al., 2014). The heterogeneity of CAFs and their different functions has yet to be explored.

1.3.2 CAFs in tumorigenesis

Accumulating evidence indicates that CAFs play a prominent role in cancer pathogenesis. The tumor-promoting mechanisms of CAFs involve the release of growth factors and cytokines, which promote tumor cell growth, angiogenesis, migration and immune modulation (Hanahan and Weinberg, 2011; Kalluri, 2016). Generally, CAFs are thought to contribute to an immunosuppressive tumor microenvironment. By releasing IL-4 and IL-8 CAFs induce differentiation of monocytes into pro-tumoral M2 macrophages that produce IL-10 and transforming growth factor β (TGF- β) (Kalluri, 2016; Takahashi et al., 2017). FAP positive CAFs have been demonstrated to oppose the Th1 response conducted via tumor necrosis factor α (TNF α) and interferon γ (INFγ) and depletion of FAP positive cells restored immune destruction of tumor cells (Kraman et al., 2010). Targeting of FAP positive cells in immune-competent cancer mouse models induced tumor cell destruction by recruitment of cytotoxic T lymphocyte (CTLs) (Wen et al., 2010). However, depletion of αSMA expressing cells induced immunosuppression and reduced survival in a murine pancreas model (Ozdemir et al., 2015). Taking CAF polarization phenotype into consideration may reveal a more accurate view of how they influence tumor immunity.

Activated fibroblast within tumors produce a plethora of factors that directly or indirectly promotes proliferation, angiogenesis, drug resistance and metastasis. Fibroblast-derived growth factors include TGFβ, fibroblast growth factor and hepatocyte growth factor, and the chemokine CXCL12. These factors stimulates cancer growth, cellular transformation and induce migratory properties of cancer cells (Bhowmick et al., 2004). The role of CAFs in angiogenesis by the release of vascular endothelial growth factor (VGEF) has been described in several studies (Fukumura et al., 1998; Pietras and Ostman, 2010). Platelet derived growth factor (PDGF) indirectly promotes angiogenesis by recruiting VEGF producing CAFs (Ferrara, 2010). Furthermore, prostaglandin E_2 (PGE_2) produced by CAFs has been found to induce VGEF expression (Amano et al., 2003). In recent years CAFs has emerged as players in the development of resistance to anti-cancer therapies (Li et al., 2015; Paraiso and Smalley, 2013). CAFs can, apart from affecting the sensitivity of cancer cells to chemo- and radiotherapy by the release of soluble factors, modulate the ECM and thereby build up a physical barrier that inhibit drug delivery. Enzymatic targeting of CAF-produced ECM in combination with chemotherapy resulted in increased overall survival (Provenzano et al., 2012).

CAFs represents an attractive therapeutic target since they, in contrast to cancer cells, are genetically stable with less risk of developing drug resistance. However the heterogeneity seen in CAFs and the increasing evidence that CAFs can work as both positive and negative regulators of cancer growth makes targeting of CAFs more complex. It has been proposed that there are several subset of polarized CAFs, much like the M1 and M2 polarized macrophages, with either tumor-suppressive effects or tumor promoting effects (Augsten, 2014). Thus a deeper understanding and characterization of CAFs is needed for successful specific targeting.

1.3.3 Cancer related inflammation

The link between cancer and inflammation was fist proposed in the19th century by Rudolf Virchow upon the observations that tumors frequently develop at sites of chronic inflammation and the presence of inflammatory cells within the tumors(Balkwill and Mantovani, 2001). Chronic inflammation affects many tumor-promoting aspects in the microenvironment(Coussens and Werb, 2002). It promotes tumor growth and survival by supplying growth and angiogenic factors. It also subvert host immunity and induce invasion and therapy resistance (Mantovani et al., 2008). In some tumors infections or inflammatory conditions precede the development of cancer. This is referred to as the extrinsic pathway in cancer inducing inflammation. In other malignancies the tumor-promoting inflammatory microenvironment is induced by oncogenic activation in which there is no underlying inflammatory conditions. This type of cancer related inflammation where transformed cells generates an inflammatory microenvironment by producing inflammatory mediators is called the intrinsic pathway (Mantovani and Pierotti, 2008). Activation of oncogenes results in constitutive production of inflammatory factors and malignant cells often overexpress pro-inflammatory mediators including chemokines, eicosanoids, cytokines, and proteases (Candido and Hagemann, 2013).

1.3.4 Immune cells in the tumor microenvironment

Various types of immune cells are frequently present within the tumor microenvironment. Cells of the innate immunity include macrophages, dendritic cells, myeloid-derived suppressor cells, neutrophils, mast cells and natural killer cells. Tumors also contain T and B-lymphocytes and cells of the adaptive immune system. Immune cells can affect malignant cells via production of growth factors, chemokines, cytokines, prostaglandins and reactive nitrogen and oxygen species. Frequently found immune cells within the tumor microenvironment includes T cells and macrophages (Grivennikov et al., 2010). Naive T cells are primed by antigen presenting cells (APC). Upon stimulation, T cells are activated and produced in large numbers. Activated T cells migrate to the site of inflammation to eliminate harmful agents. T cells are divided into two major groups: the CD8+ cytotoxic T cells (CTL) and the CD4+ T helper cells. The CTLs induce apoptosis of targeted harmful cells. Helper T cells have no cytotoxic activity and their main function is to produce cytokines that induce immune responses by other cells. Based on the cytokines they release T helper cells are divided into Th1, Th2 and regulatory T cells (T reg). Cytokines released by

Th1 cells includes IL-2, INFγ and TNFα and tends to induce pro-inflammatory responses and regulates the development and persistence of CTLs. Th2-type cytokines include IL-4 and IL-13, associated with humoral response regulation, and IL-10 and TGF-β, which regulate anti-inflammatory responses (Knutson and Disis, 2005). Unbalanced ratio of Th1 and Th2 responses are linked to a variety of inflammatory diseases including cancer (Grivennikov et al., 2010). Th2 polarized immune responses are known to promote tumor progression while Th1 responses are associated with CTL–mediated cancer cell destruction favoring tumor regression (Johansson et al., 2008). T regs are immunosuppressive cells that can down regulate T cell-mediated immunity by releasing IL-10 and TGF-β. T regs are frequently up regulated in cancer (Vignali et al., 2008).

Macrophages and dendritic cells (DC) are derived from blood monocytes. When recruited into peripheral tissues, monocytes differentiate to dendritic cell and macrophages and local microenvironmental mediators determine their phenotype (Solinas et al., 2009). Based on their function, macrophages are divided broadly into two categories: classically activated type 1 macrophages (M1) and alternative activated type 2 macrophages (M2). The names M1 and M2 were taken because they promote Th1 and Th2 responses, respectively. Classically activated M1 macrophages are driven by the Th1 cytokine IFNγ or in concert with lipopolysaccharide (LPS) (Martinez and Gordon, 2014; Sica and Mantovani, 2012). M1-macrophages are characterized by high expression of pro-inflammatory cytokines resulting in polarized type 1 immune responses and natural killer cell (NK-cell) activation (Allavena et al., 2008; Martinez and Gordon, 2014). Classically activated macrophages have high capacity to present tumor-specific antigens for propagation and anti-tumor functions of T cells. M1 macrophages phagocytize and kill target cells (Allavena et al., 2008). In contrast, M2 macrophages dampen immune responses by inducing Th2 polarization and are involved in tissue remodeling and wound healing. M2 macrophages express high levels of IL-10, TGF-β, VGEF and matrix metalloproteinases (Chanmee et al., 2014; Heusinkveld and van der Burg, 2011). Various factors such as IL-4, IL-13, PGE_2, and TGF-β have the potential to promote M2 macrophage polarization (Chanmee et al., 2014; Gordon and Martinez, 2010).

DCs are the main APC population inducing T-cell-mediated immunity. DCs transport tumor antigens to lymph nodes to activate tumor-specific T cell responses. However, impaired maturation and development of DCs by factors within the tumor microenvironment results is dysfunctional antitumor immune responses by DCs. i.e. impaired antigen-presenting abilities (Gardner and Ruffell, 2016; Zong et al., 2016). In addition, a reduced number of DC has been seen in several types of solid tumors.

1.4 PROSTANOIDS

Prostanoids are members of the eicosanoids family that represents oxidized products derived from twenty-carbon fatty acids. Prostanoids are a group of lipid mediators that are derived from arachidonic acid (AA) via the cyclooxygenase (COX) enzymatic pathway. Prostanoids

includes prostaglandins and thromboxane. Prostaglandins were first described in the 1930s, found in semen, and the prostate gland was thought to be the source. But it was first in the 1960s that prostaglandins were isolated and characterized (Bergstroem and Samuelsson, 1965; Samuelsson, 2012). In 1982 the Professors Bengt Samuelsson, Sune Bergström and John Vane was awarded with the Nobel Prize for their discoveries in this field. Prostanoids are potent biological regulators and play important roles in normal physiology and disease regulating immune functions, cardiovascular homeostasis, oncogenesis and gastrointestinal integrity (Narumiya et al., 1999; Wang et al., 2007).

1.4.1 Prostanoid biosynthesis

The prostanoid family includes prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostacyclin (PGI_2) and thromboxane (TXA_2). Prostanoids are formed from the precursor fatty acid AA that is located in cellular membranes and activated phospholipase A2 (PLA2) catalyzes its release. Free AA is then converted to the reactive intermediate prostaglandin H_2 (PGH_2) by the cyclooxygenases. There are two isoforms of cyclooxygenases; COX-1 and COX-2. COX-1 is expressed constitutively by most cells and responsible for maintaining the homeostasis of prostanoids in tissues. COX-2 on the contrary is induced upon inflammatory and oncogenic stimuli including cytokines and growth factors and is absent in most tissues under normal conditions (Wang and DuBois, 2006; Wang and Dubois, 2010; Wang et al., 2007). PGH_2 is further catalyzed into the different prostanoids by their specific synthases. The prostanoids mediate their actions in an autocrine or paracrine manner via specific G-protein coupled receptors (GPCR) (Figure 5).

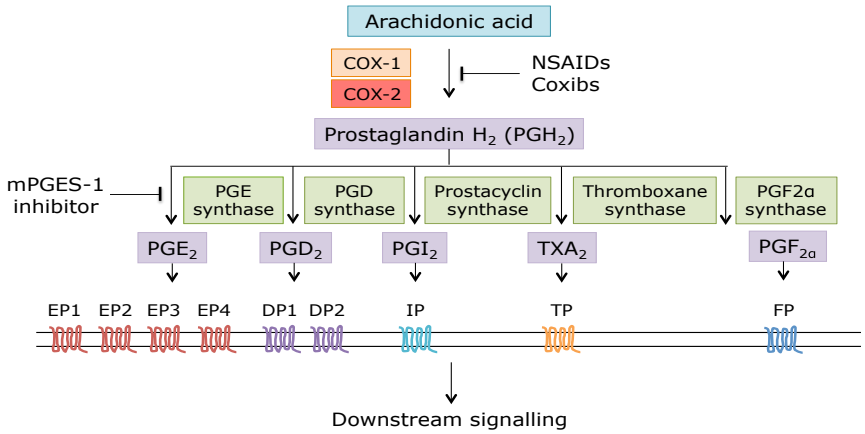

Figure 5. Schematic overview of the COX pathway and points of inhibition. Arachidonic acid is converted by cyclooxygenases (COX) to a reactive intermediate PGH_2. Terminal synthases further convert PGH_2 into prostaglandins and thromboxane. Reprinted by permission from Elsevier: Prostaglandins & Other Lipid Mediators, 2 june 2015 (10.1016/j.prostaglandins.2015.06.002) (Larsson and Jakobsson., 2015)

1.4.2 Specific prostaglandin E_2 synthases

PGE_2 is the most abundant prostanoid and is involved in essential homeostatic processes, e.g. renal functions, smooth muscle contraction, regulation of blood pressure and gastrointestinal mucosal protection (Murakami and Kudo, 2004). However, PGE_2 is produced in several pathological conditions including inflammation, fever, arthritis, tissue injury and a varietyof cancers. There are three known PGE_2 synthases. Two are membrane associated including microsomal prostaglandin E synthase 1 and 2 (mPGES-1, mPGES-2) and one is found in the cytosol and named cytosolic prostaglandin E synthase (cPGES).

PGE_2 is predominantly synthesized by mPGES-1. mPGES-1 belongs to the Membrane-Associated proteins in Eicosanoid and Glutathione metabolism (MAPEG) family and is expressed constitutively in spleen, lung, genital accessory organs and in the kidney (Jakobsson et al., 1999). In other tissues, mPGES-1 expression is low or absent under normal conditions. Expression of mPGES-1 is, like COX-2, highly inducible upon inflammatory stimuli including IL-1β, LPS and TNFα (Jakobsson et al., 1999; Larsson and Jakobsson, 2015; Uracz et al., 2002; Xiao et al., 2012). This induction suggests that mPGES-1 is crucial for PGE_2 production during inflammation.

Functionally, mPGES-1 is capable of producing PGE_2 via COX-1 and COX-2 derived PGH_2 but mPGES-1 is to some degree preferentially linked with COX-2 and in many conditions concomitantly induced with COX-2(Murakami et al., 2000). Compared to the other PGE_2 synthases, the catalytic efficiency of mPGES-1 is much higher and mPGES-1 is the only PGE_2 synthase that is induced by inflammatory stimuli (Hara et al., 2010; Jakobsson et al., 1999). The two other PGE_2 synthases is constitutively expressed and is shown to convert COX-1, but not COX-2 derived PGH_2 into PGE_2, indicating their involvement of maintaining basic levels of PGE_2 (Tanioka et al., 2000). The importance of cPGES and mPGES-2 *in vivo* is not fully understood since cPGES deficient mice were perinatal lethal and mPGES-2 deficient mice showed no specific phenotype (Hara et al., 2010).

PGE_2 is relatively stable when synthesized in *in vitro* conditions. However, *in vivo* PGE_2 is rapidly metabolized into an inactive form, 15-keto-PGE_2 by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Tai et al., 2002).

1.4.3 Prostaglandin E_2 receptors and downstream signaling

PGE_2 acts in an autocrine or paracrine manner via four different G-protein coupled receptors named EP1, EP2, EP3 and EP4. Binding of PGE_2 to its receptors results in the activation of several signal transduction pathways. Like all GPCR, EP receptors have seven trans membrane segments and each receptor is coupled to different Gα subunits. The downstream signaling pathways differ between the EP receptors depending on what family of alpha-subunit the receptor stimulates. EP1 is a Gαq coupled GPCR. Stimulation of this receptor results in activation of phospholipase C resulting in intracellular calcium release from the

endoplasmic reticulum and activation of protein kinase C. Protein kinase C triggering results in activation of the MAPK pathway promoting cell growth and activation of nuclear factor-kappa beta (NF-κβ), a transcription factor that control expression of genes responsible for both innate and the adaptive immune responses (Breyer et al., 2001; Dorsam and Gutkind, 2007; Hanahan and Weinberg, 2000). Binding of PGE_2 to EP2 or EP4 induces an increase in the levels of cyclic AMP (cAMP) due to activation of adenylatecyclase by the Gαs protein. cAMP stimulates protein kinase A activation that activates the PI3K-Akt pathway and the transcription factor cAMP–responsive element binding protein (CREB) promoting survival and growth (Dorsam and Gutkind, 2007; O'Callaghan and Houston, 2015). EP3 binding stimulates the Gαi subunit that inhibits adenylatecyclase and decreases the levels of cAMP (Breyer et al., 2001).

1.4.4 The Prostaglandin E_2 pathway in cancer

Chronic inflammation is associated with high risk of cancer development and the link between cancer and inflammation has been known for more than a century.

Approximately 15 % of malignancies are thought to be a consequence of chronic inflammation (Balkwill and Mantovani, 2001). The discovery that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) that inhibits COX-1, COX-2 or both decreased the development of malignancies and reduced tumor growth further supported the link between cancer and inflammation (Harris, 2009; Thun et al., 1991; Wang and DuBois, 2006)

PGE_2 is a key mediator in wound healing, inflammation and cancer. During tissue injury PGE_2 is produced to attract the body's immune cells and stimulate pathways important for wound healing. During normal conditions the PGE_2 levels are then decreased following resolution of inflammation. However, cancer is referred to as "a wound that never heals" and levels of PGE_2 never decline due to continuous production in the tumors.

PGE_2 is the most abundant prostanoid found in malignancies and PGE_2 plays an essential role in tumor progression and has been linked to all the hallmarks of cancer (Greenhough et al., 2009; Wang and Dubois, 2010). PGE_2 signaling via the MAPK/ERK and GSK3β-β-catenin signaling pathways induce proliferation, and activation of the PI3K-Akt pathway promotes survival (Castellone et al., 2005; Wang et al., 2005). Contribution to cancer invasiveness and angiogenesis by PGE_2 is mediated via transactivation of the epidermal growth factor receptor (EGFR) and induction of VEGF expression respectively (Buchanan et al., 2003; Fukuda et al., 2003). Recently PGE_2 was reported to induce chemo-resistance by stimulating tumor repopulation following treatment (Kurtova et al., 2015). In addition, PGE_2 is a key mediator in cancer related inflammation and mediates suppression of anti-tumor immunity (Kalinski, 2012; Nakanishi and Rosenberg, 2013)

Dysregulation of COX-2 and mPGES-1 expression resulting in higher levels of PGE_2 have been reported in a variety of solid cancers. Upregulation of COX-2 expression was first reported in colorectal cancers (Eberhart et al., 1994). Since then elevated COX-2 expression

has been linked to advanced stage disease in several malignancies including breast cancer (Boland et al., 2004; Fornetti et al., 2014; Singh et al., 2007), prostate cancer (Khor et al., 2007; Richardsen et al., 2010), lung cancer (Khuri et al., 2001) and bladder cancer (Wadhwa et al., 2005). Elevated COX-2 expression has also been associated with poor prognosis in the childhood cancers neuroblastoma (Johnsen et al., 2004) and medulloblastoma (Baryawno et al., 2008). High expression of mPGES-1 has been reported as a predictor of worse outcome in colon cancer (Sasaki et al., 2012; Yoshimatsu et al., 2001), prostate cancer (Hanaka et al., 2009), pancreatic cancer (Hasan et al., 2008), stomach cancer (Gudis et al., 2007), head and neck squamous cell carcinoma (Camacho et al., 2008), lung cancer (Hanaka et al., 2009) and in the pediatric tumor medulloblastoma (Baryawno et al., 2008). Steady state levels of PGE_2 in the body depend on the relative rates of PGE_2 biosynthesis and the PGE_2 degrading enzyme 15-PGDH. 15-PGDH converts PGE_2 into the inactive form 15-keto PGE_2. Genetic loss of 15-PGDH leads to increased levels of PGE_2 and is believed to function as a tumor suppressor (Tai, 2011). Downregulation of 15-PGDH contributes to progression of pancreatic cancer (Pham et al., 2010), lung cancer (Hughes et al., 2008), colorectal cancer (Backlund et al., 2005; Yan et al., 2004) and breast cancer (Wolf et al., 2006). Taken together, the PGE_2 pathway contains several important cancer therapeutic targets relevant for a vide variety of malignancies.

1.4.5 Prostaglandin E_2 in tumor promoting inflammation

Inflammation has emerged as a major cancer promoting factor and is now considered as one of the enabling hallmarks of cancer (Hanahan and Weinberg, 2011). Tumor promoting inflammation involves the ability of the tumor to evade host anti-tumor immunity. It is well known that interactions between malignant cells and stromal cells in the tumor microenvironment contribute and support cancer progression. Tumor cells can secrete chemokines, cytokines, and inflammatory eicosanoids that reprogram and recruit inflammatory cells to induce an immunosuppressive tumor microenvironment. Prostaglandins are key immunomodulators involved in the crosstalk of cancer cells and stromal cells in the tumor microenvironment. Generally, PGE_2 is recognized as mediator in acute inflammation were it together with other prostanoids induce vasodilation enabling recruitment of inflammatory cells to the injured tissue. However, PGE_2 also suppress mediators of acute inflammation leading to an immunosuppressive state and chronic inflammation. Thus, PGE_2 exerts both pro-inflammatory and immunosuppressive properties. In the context of cancer, the anti-inflammatory properties of PGE_2 promoting cancer immune evasion are considered to be of most importance (Figure 6).

Cancer immune evasion involves a shift from type-1 immunity to type-2 immune responses, impaired antigen-presenting cell recruitment and function, diminished cytotoxic activity of CTLs and NKs, and enhancement of immunosuppressive cells such as T regs and myeloid-derived suppressor cells. By downregulating Th1 cytokines (IFNγ, TNFα and IL-2) and upregulation of Th2 cytokines (IL-10, IL-4, and IL-6) PGE_2 helps to shift the balance away from the anti-tumor Th1 response towards immunosuppressive Th2 response in the

microenvironment (Harris et al., 2002; Snijdewint et al., 1993; Wang and Dubois, 2010)This shift results in decreased activation of CD8+ cytotoxic T cells allowing tumor cells to escape immune surveillance. PGE_2 is also known to regulate the activity of different innate immune cells.

Mononuclear phagocytes including monocytes, macrophages and dendritic cells (DCs) are frequently found within tumors. Under normal conditions mononuclear phagocytes are involved in tissue homeostasis and upon injury and infection, they clear harmful agents directly or by priming adaptive immunity and are also involved in tissue restoration (Gordon and Taylor, 2005). Together these cells have three main functions, antigen presentation, phagocytosis and cytokine production. In most solid tumors, mononuclear phagocytes are educated by the tumor microenvironment to support tumor growth and suppress anti-tumor immunity (Mantovani et al., 2008). PGE_2 seems to play a prominent role in this education. In a recent study, genetic deletion of COX in models of melanoma, breast and colorectal cancer induce a shift toward anti-cancer immunity (Zelenay et al., 2015). PGE_2 has also been shown to induce differentiation of monocytes into M2 macrophages and to induce an M1 to M2 shift in solid tumors (Heusinkveld and van der Burg, 2011; Zelenay et al., 2015).

Furthermore PGE_2 induce immune tolerance by altering the differentiation and maturation of DCs and by modulating their secretion of cytokines (Sombroek et al., 2002; Wang and DuBois, 2013). Diffrentiation of DC in the presence of PGE_2 develops a phenotype that reduces CTL and NK cell mediated immunity while promoting Th2 responses (Kalinski et al., 1999). For example, PGE_2 induces production of IL-10, a known inhibitor of DC maturation and shifts the balance from a Th1 to a Th2 response by reducing the IL-12 production (De Smedt et al., 1997; Kalinski et al., 1997; Wang and DuBois, 2013). Furthermore, PGE_2 induce T cell tolerance by up regulating CD25 and Indoleamine 2,3-dioxygenase (IDO) (Kalinski, 2012; Trabanelli et al., 2015). PGE_2 has been shown to promote development, recruitment and directly enhance the activity of suppressive immune cells such as myeloid-derived suppressor cells (Obermajer et al., 2012; Sinha et al., 2007) and regulatory T cells (Baratelli et al., 2005; Mahic et al., 2006).

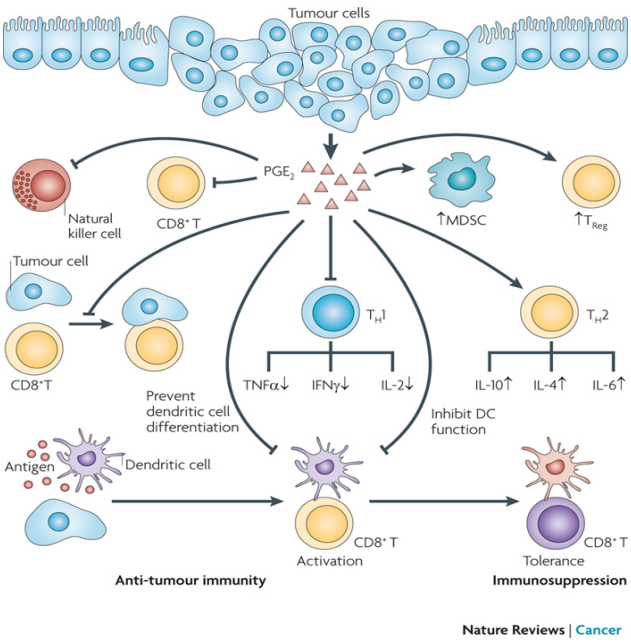

Figure 6. PGE_2 provides coordinated regulation of tumor immunosuppression. Pro-inflammatory prostaglandin E_2 (PGE_2) produced by tumor epithelial cells and/or their surrounding stromal cells induces immunosuppression through several ways, including: down regulating anti-tumor T helper 1 (Th 1) cytokines and upregulating immunosuppressive Th 2 cytokines; inhibiting $CD8^+$ T cell proliferation and activity, suppressing the anti-tumor activity of natural killer cells and stimulating the expression of regulatory T cells (Treg) and myoilid-derived suppressor cells (MDSCs); and inhibiting $CD8^+$ T cell anti-tumor functions by impairing the ability of tumor cells to directly present tumor antigen, inhibiting dendritic cell differentiation and switching the function of dendritic cells from induction of immunity to T cell tolerance. The yellow $CD8^+$ T cells have anti-tumor activity and the $CD8^+$ T cell does not have anti-tumor activity. The purple dendritic cells have the ability to present tumor antigens from tumor cells with major histocompability complex (MHC) class I molecules to activate naive $CD8^+$ T cells. The orange dendritic cells do not have the ability to activate $CD8^+$ T cells (T-cell tolerance). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, advance online publication, 10 March 2010 (doi: 10.1038/nrc2809) (Wang et al., 2010)

1.5 INHIBITION OF PROSTAGLANDIN E_2 SYNTHESIS IN CANCER

1.5.1 Nonsteroidal anti-inflammatory drugs

NSAIDs relive acute pain, fever and inflammation and are some of the worlds most widely used drugs. Beside their analgesic and anti-inflammatory effects, NSAIDs have been shown to be potent chemopreventive agents for cancer. There is abundant preclinical and clinical evidence of the benefit of regular use of NSAID in cancer prevention (Harris, 2009; Thun et al., 1991). NSAIDs target COX-1 and -2 thus inhibiting the first step in prostanoid synthesis. There are several types of NSAIDs. The traditional types of NSAIDs are non-selective and work as dual inhibitors of both COX-1 and COX-2 whereas some selectively target COX-1 or COX-2. There are several severe side effects associated with prolonged use of non-selective NSAIDs including gastrointestinal ulcers and bleeding. These side effects were thought to be caused by the disruption of the housekeeping functions of COX-1 derived

prostanoids, important for normal cellular functions in the gastrointestinal tract (Fries, 1999; Gabriel et al., 1991).

To overcome this problem, selective COX-2 inhibitors (Coxibs) was developed, e.g. celecoxib and rofecoxib. Although lower gastrointestinal toxicity was seen, the clinical trials revealed an unforeseen adverse side effect with increased risk for cardio-vascular events with the use of Coxibs (Bresalier et al., 2005; Solomon et al., 2005). The cause of cardio-vascular side effects by Coxibs is thought to be due to the resulting imbalance of PGI_2 and TXA_2 important for vascular homeostasis. PGI_2 and TXA_2 have opposing effects where PGI_2 causes vasodilation and inhibits platelet aggregation and TXA_2 promotes vasoconstriction and platelet aggregation. COX-2 selective NSAIDs inhibits PGI_2 production but allows TXA_2 synthesis that is mainly regulated by COX-1 in platelets resulting in thrombosis and cardiovascular events (Funk and FitzGerald, 2007). Due to the severe side effects, several Coxibs have been taken of the market and the use of COX-2 inhibitors has been hampered.

1.5.2 mPGES-1 inhibition

To overcome the adverse side effects but still recapitulate the cancer chemopreventive effects of NSAIDs, selective mPGES-1 inhibitors have been suggested as an alternative (Samuelsson et al., 2007). Selective inhibition of mPGES-1 and its anti-inflammatory effects have successfully been demonstrated in models of acute and chronic inflammation (Guerrero et al., 2009; Leclerc et al., 2013a; Leclerc et al., 2013b; Mbalaviele et al., 2010). Furthermore, genetic deletion of mPGES-1 in a mouse model of thrombogenesis reduced PGE_2, increased PGI_2 and, in contrast to COX-2 inhibition, did not increase thrombosis and vascular pressure (Cheng et al., 2006). The same was seen by pharmacologic inhibition of mPGES-1 which increased vasorelaxation *in vitro* by increasing PGI_2 synthesis (Ozen et al., 2017). This indicates that mPGES-1 inhibition retains anti-inflammatory properties through PGE_2 reduction while avoiding the severe cardiovascular side effects of Coxibs.

The development and screening of drugs with mPGES-1 inhibitory activity have manly been carried out in cell free conditions using mPGES-1 enzyme of human origin (Koeberle and Werz, 2015). Due to this, several mPGES-1 inhibitors failed to inhibit murine mPGES-1 activity and assessment of inhibitor efficacy *in vivo* and pre clinical studies have been hampered (Korotkova and Jakobsson, 2014). Investigation of this interspecies difference revealed three non conserved amino acids close to the active site of mPGES-1 enzyme responsible for the difference among between human and rodent enzymes(Pawelzik et al., 2010). Recently two compounds have been characterised that acts as dual inhibitors of human and murine mPGES-1. These compounds, named compound II (CII) and compound III (CIII), were shown to reduce PGE_2 levels in cell-based assays as well as in murine models of inflammation (Leclerc et al., 2013a; Leclerc et al., 2013b). Furthermore, in contrast to genetic deletion of mPGES-1 that revealed a shunting of PGH_2 towards thromboxane production,

pharmacological inhibition of mPGES-1 with compound III showed no evident shunting in the investigated cells (Leclerc et al., 2013a).

The anti cancer effect of mPGES-1 targeting has been reported in genetically knockout models of colon cancer (Nakanishi et al., 2008), breast cancer (Howe et al., 2013) and in lung cancer (Takahashi et al., 2014). Xenograft studies with stable mPGES-1 knockdown in lung and prostate cancer cell lines also showed reduced tumor growth rate (Hanaka et al., 2009). *In vivo* studies of pharmacological inhibition of mPGES-1 in xenograft models reduced growth and angiogenesis of squamous carcinoma tumors (Finetti et al., 2012). However, pharmacological studies of mPGES-1 inhibition in preclinical tumor models have been limited due to the interspecies discrepancy discussed above (Larsson and Jakobsson, 2015).

2 MATERIAL AND METHODS

Detailed materials and methods are provided in paper I-IV.

2.1 PATIENT MATERIAL

All neuroblastoma tumor tissues used in this thesis were obtained from Astrid Lindgren Children's Hospital at Karolinska University Hospital. Tissues were collected during surgery after a minimal of two weeks after any treatment and were snap-frozen and stored in –80°C. Informed consent for using tumor samples for research was obtained from parents or guardians according to the ethical approval from the Karolinska University Hospital Research Ethics Committee (approval no 2009/1369-31/1 and 03/736). Tumor and patient characteristics are summarized in **paper I**, **paper II** and **paper III**.

2.2 HUMAN CELL LINES

Human neuroblastoma cell lines used includes: SK-N-AS, SH-SY5Y, SK-N-SH, SK-N-FI, SK-N-BE (2), SK-N-DZ and IMR-32. The normal human dermal fibroblast cell line NHDF was used in **paper IV**. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100µg/mL streptomycin and 100 IU/mL penicillin.

2.3 EX VIVO AND IN VITRO ANALYSIS

2.3.1 RNA expression

Reverse transcription-PCR was used in paper I to analyze receptor expression in neuroblastoma cell lines. Quantitative Real-Time PCR Analysis was preformed to detect relative mRNA levels in primary neuroblastoma tumor in **paper II** and in in vitro models of neuroblastoma in **paper IV**. Gene expression and impact on overall survival of neuroblastoma patients was performed using the publicly available database R2: microarray

analysis and visualization platform (r2.amc.nl). mRNA from 88 human neuroblastoma samples, included in the Versteeg-88 dataset, was analyzed in **paper II**.

2.3.2 Protein detection

Immunohistochemical analysis was used to assess protein expression patterns and levels. In **paper I** formalin-fixed and paraffin-embedded tumor tissue sections were used and in **paper II**, **paper III** and **paper IV** snap frozen samples of tumor tissue or collected spheroids were sectioned. To preserve tissue morphology collected fresh tissue from experimental tumors or spheres was embedded, without fixation, in a cryomold with Tissue-Tech O.C.T. Compound. Fluorescence immunocytochemistry was preformed to study protein expression and induction in *in vitro* experiments with neuroblastoma cell lines in **paper I** and **paper IV**. Cytokine arrays was used to analyze relative cytokine expression in supernatants from MCTS of SK-N-AS and NHDF cultivated together with LPS and IFNγ stimulated PBMCs or non-stimulated PBMCs in **paper IV**. Western blotting was preformed to detect relative protein levels in neuroblastoma cell lines (**paper I**), primary neuroblastoma tumors (**paper II**) and in experimental tumors (**paper III**).

2.3.3 Flow cytometry

Flow cytometric analysis of single cell suspensions of experimental tumors and spleen from mice treated with the selective mPGES-1 inhibitor compound III (CIII) was performed in **paper III**. Tissues were dissociated mechanically and red blood cells were lysed before staining. All antibodies used are listed in **paper III**.

2.3.4 Cell viability assays

Cell viability of neuroblastoma cell lines in **paper I** was investigated using MTT-assays. The proportion of viable cells in MCTS, cultivated with neuroblastoma cells (SK-N-AS) and fibroblasts (NHDF), were quantified by measuring cellular ATP content using the CellTiter-Glo 3D Cell Viability Assay in **paper IV**.

2.3.5 Receptor activation

In **paper I**, intracellular levels of calcium and cAMP in response to PGE_2 treatment was analyzed. Intracellular mobilization of calcium in neuroblastoma cell lines was visualized and measured by using the fluorescent dye Fluo-4/AM in a confocal laser-scanning microscope. The intracellular levels of cAMP were determined by using a cAMP ELISA EIA kit.

2.3.6 Migration assay

To analyze the migratory effect of mPGES-1 inhibition on fibroblasts (NHDF) co-cultured with neuroblastoma cells (SK-N-AS) the trans-well Boyden Chamber Cell Migration Assay was used in **paper III**.

2.3.7 Prostanoid measurements and pharmacokinetics

In order to study the pharmacokinetics of CIII in **paper III** a targeted LC-MS/MS method was established. The levels of CIII was analyzed in plasma, tumor tissue and spleen collected 2h, 4h, and 6h post intra peritoneal injection. Prostanoid levels in neuroblastoma cell lines in **paper I**, in primary neuroblastoma tumors in **paper II** and in experimental tumors in **paper III** was determined using LC-MS/MS. To analyze levels of PGE_2 production by MCTS in **paper IV** we used prostaglandin enzyme immunoassay (EIA).

2.4 IN VIVO EXPERIMENTS

2.4.1 Xenograft mouse models

Athymic female NMRI nu/nu mice were kept in pathogen free condition given food and sterile water in *ad libitum*. In **paper II** and **paper III** we subcutaneously injected neuroblastoma cells (SK-N-AS) on the flank of 4-8 week old mice. Tumor volume and the weight of the mice was measured every day post cell injection. The experiments were approved by the regional ethics committee for animal research (approval N231/14) in accordance with the Animal Protection Law (SJVFS 2012:26).

2.4.2 TH-MYCN transgenic mouse model

The transgenic TH-MYCN mice spontaneously develop an aggressive form of neuroblastoma. Expression of a MYCN construct is coupled to the tyrosine hydroxylase promotor expressed by neuroectodermal cells during the development of the sympathetic nervous system (Weiss et al., 1997). All homozygous mice develop tumors within 7 weeks (Rasmuson et al., 2012). The animals were kept in ventilated cages with an enriched environment and food provided *ad libitium*. In **paper III** we randomized homozygous animals into treatments group at the age of 4,5 weeks when small tumor lesions are present. All transgenic animal experiments were approved by the regional ethics committee for animal research (ethical permit N26/11 and N42/14) in accordance with the Animal Protection Law (SFS1988:534).

2.5 STATISTICS

For statistical comparisons between two independent groups we used unpaired t-test or Mann-Whitney test when applicable. Statistical differences of several independent groups were analyzed by two-way ANOVA. Kaplan-Meier estimator was used for survival analysis and the significance was calculated using Log-rank test (Mantel-Cox). Graphs and statistics was prepared and analyzed in GraphPad prism.

3 AIMS

The general aim of this thesis was to assess the importance of PGE_2 in neuroblastoma and to characterize mPGES-1 as a potential novel therapeutic target in neuroblastoma.

Specific aims:

Paper I. To investigate PGE_2 signaling in neuroblastoma cell lines.

Paper II. To study the significance of mPGES-1 in primary neuroblastoma.

Paper III. To investigate the effect of pharmacological mPGES-1 inhibition in pre-clinical neuroblastoma mouse models.

Paper IV. To establish an *in vitro* preclinical model simulating the neuroblastoma microenvironment, enabling mPGES-1 inhibition in combination with conventional cancer therapies.

4 RESULTS AND DISCUSSION

4.1 PAPER I: PGE_2 SIGNALING IN NEUROBLASTOMA

Prostaglandin E_2 (PGE_2) is known to promote tumor related inflammation (Kalinski, 2012; Wang and Dubois, 2010). Selective COX-2 inhibition was shown to reduce neuroblastoma growth *in vitro* and *in vivo (*Johnsen et al., 2004; Ponthan et al., 2007*)*. In this paper we therefore wanted to investigate the significance of PGE_2 signaling in neuroblastoma.

The first step to evaluate the importance of PGE_2 signaling in neuroblastoma was to study the expression of the specific PGE_2 receptors EP1, EP2, EP3 and EP4. Immunohistochemical staining of 28 primary neuroblastoma tumors with different clinical background showed expression of all receptor subtypes. Further, expression of the receptors was analyzed in a panel of 7 different neuroblastoma cell lines. Expression of all the receptors was determined at the mRNA level with RT-PCR and at the protein level with immunofluorescence. No evident difference in expression pattern was recognized between clinical subsets of neuroblastoma neither in primary tumors nor in cell lines.

Next, we evaluated the ability of neuroblastoma cells to synthesize PGE_2. PGE_2 levels were measured using LC-MS/MS. SK-N-SH cells did not produce detectable levels of PGE_2 whereas PGE_2 was found to be produced by the MYCN- amplified cell line SK-N-BE (2) in low levels under normal growth conditions. Addition of AA, a substrate for prostanoid synthesis, to the culturing media resulted in increased production of PGE_2 in SK-N-BE (2) cells but not in SK-N-SH. However arachidonic acid in combination with the inflammatory cytokine IL-1β, a known inducer of both COX-2 and mPGES-1, did induce PGE_2 production in both cell lines. These findings indicated that the PGE_2 synthesis pathway in neuroblastoma cell lines is triggered upon inflammatory stimuli.

Previous studies have shown that COX-inhibitors impede neuroblastoma cell growth (Johnsen et al., 2004). This and the knowledge that neuroblastoma cells express all the EP receptors made us examine the direct effect of PGE_2 on cell growth. Increasing concentrations of PGE_2 was added to the culturing media of serum starved cells and cell viability was measured using 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 24, 48, 72 and 96 hours. PGE_2 significantly increased neuroblastoma cell viability in a dose and time dependent manner. Furthermore, we could rescue neuroblastoma cells from celecoxib-induced cytotoxicity by adding PGE_2. This shows that PGE_2 has a direct effect on neuroblastoma cell growth and not only supports tumor growth by contributing to a cancer-promoting inflammatory microenvironment. An increase in cell proliferation by PGE_2 has also been demonstrated in medulloblastoma, another neural embryonic tumor (Baryawno et al., 2008). The proliferative effect of PGE_2 on cancer cells has mainly been reported in cancer of epithelial origin.

To confirm PGE_2-induced receptor activation and to assess the mitogenic mechanism behind the observed increase in neuroblastoma cell viability, we analyzed intracellular downstream second messengers and effectors upon PGE_2 activation.
EP1 activation results in mobilization of intracellular calcium and by using fluorescent calcium dye Fluo-4/AM we could visualize a rapid increase in cytoplasmic calcium levels when adding PGE_2 to neuroblastoma cells. Since EP2, EP3 and EP4 acts via adenylate cyclase and cAMP we treated neuroblastoma cells with PGE_2 and determined the intracellular levels of cAMP. EP2 and EP4 signals via Gαs-protein that stimulates cAMP production while EP3 is linked to Gαi-protein inhibiting cAMP production. After 20 minutes of PGE_2 incubation a net response of increased cAMP was observed. A Gαs protein inhibitor could prevent this effect.

PGE_2 have been reported to induce survival, proliferation and invasion via activation of several mitogenic pathways in carcinomas including the Ras-Erk, GSK3β- β catenin and PI3K-Akt pathway (Wang and Dubois, 2010). Western blotting of PGE_2 treated neuroblastoma cells showed a time dependent increased in phosphorylated Akt, a protein involved in cancer cell survival and proliferation. The PI3K-Akt pathway is the one of the most common and potent survival signaling cascades in cancer (Vivanco and Sawyers, 2002). Previous, phosphorylated Akt has been linked to decreased overall survival in neuroblastoma (Opel et al., 2007) and another study found Akt and mTOR to be activated in all tumors in a panel of 30 primary neuroblastomas while no activation was found in non-malignant adrenal glands (Johnsen et al., 2008). Here we show PGE_2 to be an activator of Akt in neuroblastoma resulting in increased cell survival.

EP receptor targeting has been proposed as a possible alternative to COX-inhibition. We wanted to evaluate this possibility and elucidate if any of the receptors are of greater importance for neuroblastoma growth. We treated six different neuroblastoma cell lines with

a panel of EP- receptor antagonists and evaluated cell viability. All the antagonists reduced neuroblastoma cell growth and the drugs selectively targeting EP1, EP3 and EP4 were most effective in reducing cell viability. This suggests that more than one receptor is important in neuroblastoma growth. However, it is difficult to draw conclusions of the importance of specific receptors since receptor affinity, drug specificity and levels of receptor expression should be taken into consideration. Several preclinical studies with EP receptor antagonists have been successful in hampering tumor growth (O'Callaghan and Houston, 2015).

Comparison of EP receptor expression using public available expression arrays of neuroblastoma (http://r2.amc.nl, R2) shows that high expression of EP2 have the worst impact on overall survival of neuroblastoma patients. At the time of this study selective EP2 antagonists was not commercially available and most research of EP2 receptor have been conducted in EP2-deficient mice. However, a novel selective EP2 receptor antagonist has shown promising results in cancer studies (af Forselles et al., 2011; Aoki and Narumiya, 2017; Jiang and Dingledine, 2013). This enables further studies of the impact of PGE_2-EP2 signaling in neuroblastoma. Furthermore, several studies have proven an existing transactivation of EGF receptor via EP receptors, adding further complexity to the PGE_2 signaling pathway (Han et al., 2006; Oshima et al., 2011; Pai et al., 2002). Whether combinational treatment of EP antagonist with different receptor selectivity could improve the anti-cancer effect is yet to be elucidated.

Taken together our results demonstrate that PGE_2 production can be induced in neuroblastoma cells, and that the EP receptors are abundantly expressed and activated by PGE_2 resulting in cancer survival signaling cascades, hence creating an autocrine or a paracrine survival loop for neuroblastoma (Figure 7). This suggests that selective targeting of PGE_2 signaling and production could be an alternative therapeutic approach in neuroblastoma that potentially could avoid possible side effects of COX-inhibitors.

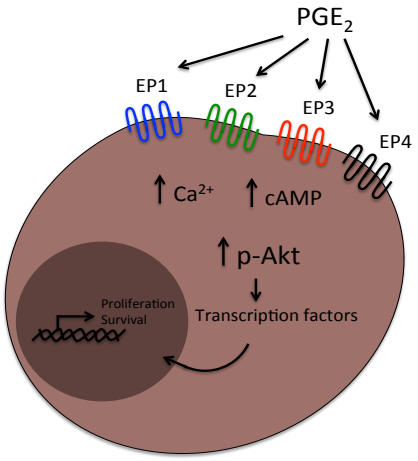

Figure 7. Simplified illustration of PGE_2 signaling in neurblastoma. Neuroblastoma cells express all EP receptors. EP receptor activation by PGE_2 results in downstream phosphorylation of Akt and activation of survival signaling cascades.

4.2 PAPER II: MPGES-1 IN NEUROBLASTOMA

PGE_2 is known to influence cells in the tumor by subverting host anti-tumor responses promoting an immunosuppressive tumor microenvironment. Although many adult cancers are presented with an inflammatory microenvironment, the knowledge is limited on importance of inflammation in childhood malignancies. In this study, we focused on the inflammatory COX/mPGES-1/PGE_2 pathway in different clinical subsets of neuroblastoma.

We first analyzed mRNA expression levels of mPGES-1, COX-1 and COX-2 in primary neuroblastoma tumors of different subtypes. The high-risk group included *MYCN*-amplified and 11q-deleted tumors. The low-risk group included tumors that had been defined as L or MS according to the International Neuroblastoma Risk Group staging system (INRGSS). The relative expression of mPGES-1 and COX-1 was found to be significantly higher in the 11q-deleted tumors compared to the low-risk group. The increased expression of mPGES-1 seen in the 11q-deleted tumors was validated by mPGES-1 expression analysis using public gene expression platforms showing that high levels of mPGES-1 expression correlated with reduced overall survival in high-risk neuroblastoma. Protein expression of synthases involved in prostaglandin synthesis was analyzed by immunohistochemistry. Quantification of protein levels of mPGES-1, COX-1 and COX-2 confirmed the mRNA levels showing significantly higher levels of mPGES-1 in the 11q-deleted tumors. COX-1 protein expression was comparatively higher than COX-2 expression in the samples. However, no significant differences were found in COX-1 and COX-2 protein levels between the different subgroups. Surprisingly overall low levels of COX-2 was detected in the analyzed neuroblastoma samples. A previous study has described neuroblastoma with high COX-2 expression (Johnsen et al., 2004). Comparison of COX-2 expression in this study was done between malignant tissue and non-malignant adrenal glands whereas we compared differences within subgroups of cancerous tissues that partly could explain these discrepancies.

Next, we evaluated the actual levels of prostaglandins in neuroblastoma tissues. Prostaglandins were measured with LC-MS/MS in a panel of 29 primary neuroblastoma tumors. Significantly higher levels of PGE_2 were found in 11q-deleted tumors compared to both *MYCN*-amplified tumors and low-risk tumors. Interestingly 15-PGDH, the PGE_2 degrading enzyme was expressed at significantly lower levels in 11q-deleted tumors. The balance between the activity of PGE_2 synthases and 15-PGDH determines the level of PGE_2 production. In 11q-deleted neuroblastomas the high expression of mPGES-1 and the low expression of 15-PGDH plausible causes the high levels of PGE_2. Down regulation of 15-PGDH have been described to contribute to pathological levels of PGE_2 in a variety of malignancies and 15-PGDH has been proposed to act as a tumor suppressor (Tai, 2011). 15-PGDH have been shown to be down regulated by epigenetic repression (Backlund et al., 2008; Lodygin et al., 2005). It would be of interest to evaluate the transcriptional regulation of 15-PGDH in neuroblastoma and assess 15-PGDH as a possible therapeutic target.

Patients with 11q-deletions are often older at disease onset and have slower disease progression compared to patient with *MYCN*-amplified tumors. A recent analysis showed that

children with non-*MYCN* amplified high-risk tumors that are older than 18 months at diagnosis have higher expression levels of inflammatory genes (Asgharzadeh et al., 2012).Together with our results this suggests that high-risk 11q-deleted neuroblastoma tumors from older children presents a more inflammatory phenotype.

Tumors with high expression of mPGES-1 resulting in elevated levels of PGE_2 are mostly carcinomas, cancer with epithelial origin (Nakanishi et al., 2010). An exception is the embryonic tumor medulloblastoma that, like neuroblastoma originates from neural cells, also express high levels of mPGES-1 (Baryawno et al., 2008). However, in these tumors mPGES-1 is predominantly expressed by neoplastic cells. Unexpectedly, immunohistochemical analysis of mPGES-1 protein expression in neuroblastoma tumors revealed a staining pattern resulting from stromal cells or infiltrating cells rather than tumor cells. To confirm this a double staining with antibodies against mPGES-1 and GD2, a marker for tumors of neuro ectodermal origin, was performed. A clear discrepancy in staining pattern was seen indicating that PGE_2 in the tumors are produced by cells in the tumor stroma and not by the tumor cells. Most cells of the body can produce PGE_2. However, epithelial cells, infiltrating immune cells and fibroblasts present the major sources of PGE_2 in inflammatory responses.

Dual labeling with mPGES-1 and the endothelial marker CD31 exhibited no co-expression. To further localize the cellular origin of mPGES-1 expression and to consider the level of PGE_2 signaling and inflammation in the tumor microenvironment we analyzed immune cell markers together with mPGES-1. Tumors from the three different subsets were analyzed. Macrophages are known to express mPGES-1 (Westman et al., 2004). Abundant expression of both the macrophage marker CD68 and M2 polarized macrophages marker CD163 was evident in all tumors analyzed. However, no co-localization was found between mPGES-1 and the macrophage markers. To evaluate the degree of macrophage infiltration and polarization we quantified expression of M1 and M2 markers in tumors. The staining revealed a shift towards an M2 polarization phenotype in the high-risk tumors compared to low-risk tumors. Neuroblastoma have been described to contain an immunosuppressive microenvironment hampering anti-tumor immunity (Pistoia et al., 2013). Our results correspond with the findings by Asgharzadeh and colleges revealing significantly greater numbers of CD163 positive cells in high-risk metastatic tumors compared to low-risk tumors (Asgharzadeh et al., 2012).

Other myeloid cells, which apart from being directly regulated by PGE_2 have been reported to actively produce PGE_2 are dendritic cells and MDSCs (Fogel-Petrovic et al., 2004; Serafini, 2010). To assess mPGES-1 expression by dendritic cells and to cover all myeloid cells double staining of mPGES-1 together with CD11c and CD11b was preformed. None of the dendritic- or myeloid- cell markers co-localized with mPGES-1. However, CD11c expressing dendritic cells was found exclusively in areas with mPGES-1 expressing cells. Next we studied lymphocyte infiltration. T-cells were present in all investigated tumors in proximity to mPGES-1 positive cells. Interestingly, only the tumors in the low risk group that spontaneously regress showed abundant presence of B-cells.

To assess whether the mPGES-1 expressing cells were of mesenchymal origin we preformed dual labeling with mPGES-1 and vimentin. The majority mPGES-1 expressing cells in *MYCN*-amplified and low risk tumors did express vimentin. However, vimentin was widely expressed in the tumors and not exclusively expressed by mPGES-1 positive cells. Since mPGES-1 did coincide with the mesenchymal marker vimentin and since fibroblasts have been reported to produce PGE_2 we analyzed mPGES-1 expression together with a panel of CAF markers including αSMA, PDGFRβ, PDGFRα, FAP and FSP-1. In the tumors evaluated, expression of mPGES-1 was found to overlap with one or more markers of CAFs. Our results are in line with findings in a recent study of head and neck squamous carcinoma, where cancer cells induced fibroblasts to express mPGES-1 and release PGE_2 (Alcolea et al., 2014). There is accumulating evidence that fibroblasts within the tumor play an important role in cancer pathogenesis. CAFs have been shown to promote an immunosuppressive microenvironment and have even been suggested as targets for immunotherapy. One recent study shows that CAFs are educated by cancer cells and contributes to tumor enhancing inflammation via NF-κβ (Erez et al., 2010), a transcription factor implied in regulation of mPGES-1 expression (Bage et al., 2010; Diaz-Munoz et al., 2010).

Finally, we evaluated the effect of PGE_2 inhibition on neuroblastoma growth in a preclinical 11q-deleted xenograft model. Tumor bearing mice were treated with the non-selective COX inhibitor diclofenac. This resulted in a significant reduction of tumor growth and decreased PGE_2 levels.

In conclusion, we show that high-risk neuroblastoma tumors present an immunosuppressive microenvironment and that the inflammatory COX/mPGES-1/PGE_2 pathway is highly activated in 11q-deleted neuroblastomas. In addition, our findings suggest that cancer-associated fibroblasts and PGE_2 might play a role in the complex tumor-stroma interactions within the tumor microenvironment and promote neuroblastoma-related inflammation.

4.3 PAPER III: MPGES-1 INHIBITION IN NEUROBLASTOMA

In this study we aimed to investigate specific effects of pharmacological mPGES-1 inhibition in PGE_2 producing CAFs using pre-clinical *in vivo* models of high-risk neuroblastoma. To mimic high-risk 11q-deleted tumors we xenografted human SK-N-AS cells with confirmed 11q-deletion in NMRI mice. We also used the MYCN-driven transgenic TH-*MYCN* mouse model. These animals spontaneously develop an extremely aggressive form of neuroblastoma resembling human *MYCN*-amplified tumors (Rasmuson et al., 2012; Weiss et al., 1997). Expression of mPGES-1 in both *in vivo* tumor models was detected exclusively in PDGFRβ expressing cells, a marker for CAFs, and not in malignant cells similar to what we previously described in human neuroblastomas. Interestingly, STAT3, one of the major regulators of tumor-promoting inflammation was activated in mPGES-1 expressing cells (Yu et al., 2009).

Since we in paper I and in paper II revealed an active PGE_2 pathway in neuroblastoma we investigated the expression of PGE_2 receptors in the two *in vivo* models and in corresponding primary human tumors. All receptors subtypes were abundantly expressed as seen in paper I.

EP4, however, was predominantly expressed in the stromal cells of both the 11q-deleted and *MYCN*-amplified tumors. The expression pattern of the receptors in the model tumors corresponded to the primary high-risk neuroblastoma subsets. This suggests that PGE_2 production and signaling is conducted in the same manner in primary human neuroblastomas and in the two corresponding mouse models. Taken together this underlines the relevance of these preclinical neuroblastoma models for pharmacological targeting of mPGES-1.

There are phenotypic differences between the human and murine mPGES-1 enzyme. Three amino acids differ leading to inhibitors developed to the human enzyme inefficient towards murine mPGES-1 (Pawelzik et al., 2010). Recently the small molecule inhibitor Compound III (CIII) was characterized as a selective dual human/rodent mPGES-1 inhibitor with efficacy in both *in vitro* and *in vivo* murine models of inflammation but have never investigated as an anti-cancer agent (Leclerc et al., 2013a; Olesch et al., 2015). In order to study the pharmacokinetics of CIII a targeted LC-MS/MS method was established. A rapid uptake of CIII was revealed with the highest concentration in plasma and tumor tissues at around 2 h post injection. The reduction of PGE_2 levels follows the concentration of CIII with the highest effect on PGE_2 inhibition 2 h after administration. We did not detect any other prostanoids in the tumors and expression of mPGES-1 and COX-1 in tumor tissue were unchanged with CIII treatment.

The therapeutic effect of the mPGES-1 inhibitor CIII on neuroblastoma growth was then assessed in the two models of high-risk neuroblastoma. Mice with xenografted 11-q deleted tumors were treated daily with CIII (50 mg/kg) either as a prophylactic treatment from the day of tumor cell injection or as a treatment of established tumors, starting at a tumor volume of 0.2 ml. The early initiated prophylactic treatment did not delay early tumor establishment, however the treatment significantly hampered the development of large macroscopic tumors with a volume of ≥1 ml. Treatment of established tumors, starting at 0.2 ml, significantly reduced tumor volume compared to the untreated control group. Clearly, the daily transient reduction in PGE_2 levels had a distinct impact on tumor growth. Even though the prophylactic treatment with CIII did not delay tumor formation, the aggressive growth rate seen in untreated mice once tumors were established was significantly reduced. Prophylactic treatment with CIII and treatment of established tumors resulted in the same tumor weight at sacrifice. This could mean that mPGES-1 targeting at an early time point promotes a less malignant microenvironment hampering tumor growth. At the same time, targeting of mPGES-1 in an established PGE_2 dependent and immunosuppressive tumor milieu might be sufficient to halt neuroblastoma growth.

Next we evaluated the effect of selective mPGES-1 inhibition on tumor growth in fully immune competent TH-*MYCN* transgenic mice. Homozygous TH-*MYCN* transgenic mice were treated daily with CIII (50mg/kg) from the age of 4.5 week, an age when only small tumor lesions are present (Carlson et al., 2013), until 6 weeks of age. Tumor growth was significantly reduced in CIII treated mice.

To compare effect of selective mPGES-1 inhibition with non-selective COX inhibition on neuroblastoma growth we treated homozygous TH-*MYCN* mice with diclofenac in the drinking water (10 mg/L) for 14 days. The tumor weight reduction was comparable with effect seen by CIII treatment. COX inhibition is widely studied and has great potential foremost as preventive treatment in adult cancer (Wang and Dubois, 2010).PGE_2 inhibition envisioned great promise in combination with conventional cancer treatments such as radiation therapy (Brocard et al., 2015) and chemotherapy(Ponthan et al., 2007). PGE_2 inhibition also proved to reduce chemo resistance (Kurtova et al., 2015). In a recent report a synergistic effect of COX inhibition and PD-1 blockade in *in vivo* models of melanoma, breast and colorectal cancer was shown (Zelenay et al., 2015). However, the severe side effects associated with COX inhibitors on both the gastrointestinal tract and the cardiovascular system has limited their clinical use. The TH-*MYCN* model is known for its extremely aggressive growth and it is difficult to halt tumor progression once the tumor is established (Eissler et al., 2016; Rasmuson et al., 2012). We were able to suppress the tumor growth with a single treatment, seemingly without any toxic side effects, targeting the tumor-stroma interaction. With pharmacological mPGES-1 inhibition we would recapitulate the anti-tumor benefits of COX inhibition without the side effects caused by the systemic and unspecific reduction of all prostanoids.

PGE_2 contributes to an immunosuppressive milieu leading to inhibition of anti-tumor immunity (Nakanishi and Rosenberg, 2013; Sinha et al., 2007; Wang and Dubois, 2010). Neuroblastoma presents an immunosuppressive microenvironment (Borriello et al., 2015; Pistoia et al., 2013) and high-risk neuroblastoma has been described with higher infiltration of alternatively activated macrophages (M2) compared with loco regional neuroblastoma (Asgharzadeh et al., 2012). Furthermore, the tumor microenvironment of TH-*MYCN* mice promotes a transition of macrophages towards a tumor promoting M2 phenotype during tumor progression (Carlson et al., 2013; Mao et al., 2016). We therefore analyzed macrophage polarization markers, CD86 (M1) and CD206 (M2) in tumor and spleen from mice treated with CIII to assess the immunomodulatory effect of mPGES-1 inhibition.

Immunohistochemical staining revealed a decrease in tumor-promoting M2 macrophages in the CIII treated tumors. In addition, flow cytometry analysis showed a significant shift towards M1 polarization while total macrophage frequencies remained unchanged. This demonstrates that mPGES-1 targeting support anti-tumor immunity via macrophage education toward an M1 phenotype.

Angiogenesis is a rate-limiting factor in tumor development and progression. The PGE_2 pathway is known to promote angiogenesis and COX-2 inhibition has anti-angiogenic properties in neuroblastoma (Jain et al., 2008; Liu et al., 2015; Ponthan et al., 2007). We therefore analyzed the impact of new vessel formation by mPGES-1 inhibition using immunohistochemistry. A significant decrease in CD31 expression, an endothelial cell marker, was seen in TH-*MYCN* tumors with CIII. This indicates that selective mPGES-1

inhibition can recapitulate the anti-angiogenic effect of COX-2 inhibition and that treatment at an early state hampers angiogenesis and thereby tumor development.

Lately several reports demonstrate that CAFs promote an immunosuppressive tumor microenvironment via M2 polarization. Since PDGFRβ positive CAFs in the experimental tumors express mPGES-1 and the immune regulator STAT3 we analyzed the amount of PDGFRβ positive cells in CIII treated tumors. CIII treatment resulted in less PDGFRβ positive cells in the TH-*MYCN* tumors and in the prophylactic treated xenograft tumors. There was no difference in tumors from mice that received CIII after tumor establishment. This suggests that CIII targeting not only directly inhibits PGE_2 synthesis but also, in the developing tumor microenvironment, indirectly reduce PGE_2 and thereby alters the tumor malignancy.

Finally, we investigated the impact of PGE_2 signaling in tumor-promoting recruitment of fibroblasts *in vitro*. One important inducer of mPGES-1 is IL-1β (Jakobsson et al., 1999) and the receptor IL-1 receptor type I (IL-1RI) coincided with mPGES-1expressing PDGFRβ positive cells in experimental tumors. *In vitro*, IL-1β induced mPGES-1 expression and migration of human dermal fibroblasts towards neuroblastoma SK-N-AS cells in a transwell assay. Fibroblast migration was reduced in the presence of CIII or an EP4 antagonist, suggesting that PGE_2 plays a role in fibroblast migration and infiltration in high-risk neuroblastoma.

Taken together our results demonstrate that pharmacologic mPGES-1 inhibition alters the tumor microenvironment and hampers tumor progression in neuroblastoma (Figure 8).

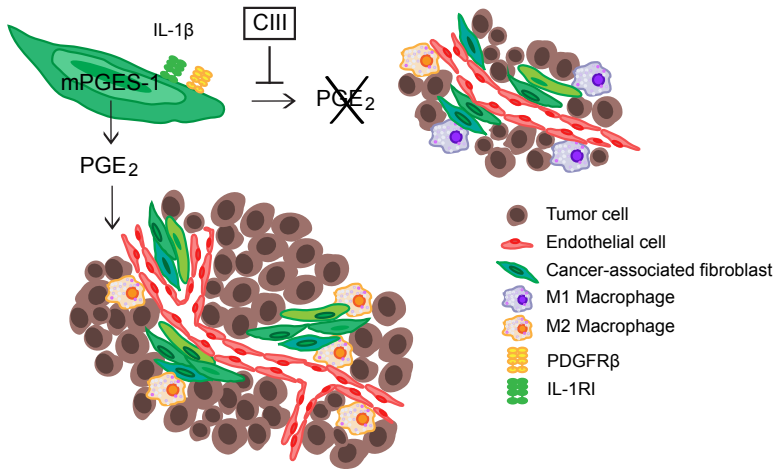

Figure 8. Illustration of the role of PGE_2 in neuroblastoma. CAFs residing in the tumor express PDGFRβ and IL-1RI. IL-1β in the tumor microenvironment stimulates mPGES-1 expression and leads to PGE_2 production. PGE_2 release from the CAFs leads to increased angiogenesis, an immunosuppressive microenvironment and tumor growth. By inhibiting mPGES-1 with CIII the actions of PGE_2 is reversed and the tumor growth halted. The illustration is from **paper III**.

4.4 PAPER IV: MPGES-1 INHIBITION IN COMBINATION WITH CONVENTIONAL CANCER THERAPIES

Tumorigenesis has generally been looked upon as a cell-autonomous activity where genetic events result in cell transformation and neoplastic growth. Today, however, the significance of stromal cells populating the tumor microenvironment that promote tumor growth and development is well established (Bissell and Radisky, 2001).

In paper II we show that high-risk neuroblastoma presents an immunosuppressive microenvironment and fibroblasts within the tumors express mPGES-1. In paper III PGE_2 targeting by pharmacological inhibition of mPGES-1 resulted in a less tumor-promoting microenvironment and reduced neuroblastoma growth. In this study we aimed to establish a preclinical *in vitro* model simulating the neuroblastoma microenvironment. This would enable further understanding of the complex cellular interactions and provide us with a screening tool for targeting PGE_2 driven tumor-stroma interaction in combination with conventional therapies that mainly targets transformed cancer cells.

Evaluation of mPGES-1 expression in monocultures of fibroblasts and co-cultures of fibroblasts and neuroblastoma cells in monolayer cultures revealed no induced mPGES-1 expression without IL-1β stimulation.

Important functions of cells seen in tissues are lost when the cells are forced to grow as monolayers in culture. In contrast, three dimensional (3D) cultures retain many cancer tissue features including hypoxic gradients, cells in different metabolic and proliferative state, limited drug penetration and improved cell-cell contact (Pampaloni et al., 2007). In order to recapitulate the pathobiology described in neuroblastoma we decided to establish multicellular tumor spheroids (MCTS) by co-culturing fibroblasts and neuroblastoma cell lines in non-adherent conditions. By doing so we could retain *in vivo* features with a proliferative and hypoxic gradient. We could also detect an induced mPGES-1 expression not seen in 2D co-cultures underlining the importance of the *in vivo* characteristics seen in 3D models.

In order to analyze the phenotype of fibroblasts grown together with neuroblastoma cells in MCST we preformed immunohistochemistry with mPGES-1 and a panel of CAF markers. mPGES-1 expression coincided with markers of CAFs in the MCTS in agreement with mPGES-1 expression in primary tumors and experimental tumors of neuroblastoma. The fibroblast population expressed FAP, vimentin, FSP-1, PDGFRβ and PDGFRα. However, the marker αSMA, a myofibroblasts marker expressed by activated fibroblast during wound healing was only weakly expressed. Accumulating reports describe fibroblasts within the tumor microenvironment as a heterogenic cell population with differently polarized subpopulations (Augsten, 2014; Sugimoto et al., 2006). It is generally thougt that CAFs are related to myofibroblasts. However, all fibroblasts within the tumors do not express αSMA but these cells are still thought to possess tumor promoting abilities (Erez et al., 2010). Interestingly, fibroblasts within MCTS expressing mPGES-1 also expressed IL-1RI similar to

the experimental tumors. IL-1β is one of the main inducer of mPGES-1 and cancer cells have been shown to activate fibroblasts to induce tumor-promoting inflammation in response to IL-1β stimulation (Erez et al., 2010).

By growing tissue-derived tumor spheroids (TDTS) generated by partial enzymatic dissociation of tumor tissue we wanted to evaluate if we could preserve the phenotype of stromal cells that have been educated in an authentic microenvironment. Characterization of the TDTS showed a clear proliferative gradient and in resemblance to *in vivo* experimental tumors mPGES-1 coincided with PDGFRβ as described in Paper III. However, the ratio of fibroblasts and cancer cells in the TDTS did not correspond to the tumor tissue and is in need of optimization.

COX-2 positive cells in both 3D models were found in the hypoxic area at the border of the necrotic core and the proliferative zone, an area known to harbor quiescent and senescent cells. COX-2 expression is known to be induced by hypoxia (Zhao et al., 2012). Since chemo- and radiotherapy targets actively dividing cells, senescent and quiescent cells within a tumor are thought to be more resistant. The COX-2 expressing cells in the MCTS might represent a chemo resistant cell population and yet another interesting therapeutic target for combinational studies. However this needs to be further assessed.

Since the fibroblast population in the MCTS expressed mPGES-1 we evaluated the combinational effect of selective mPGES-1 inhibition and the conventional chemotherapeutic drugs doxorubicin and vincristine commonly used in the treatment of high-risk neuroblastoma. Single treatment of the mPGES-1 inhibitor CIII reduced PGE_2 levels and significantly reduced the cell viability in the MCTS. mPGES-1 inhibition with CIII augmented the cytotoxic effect of doxorubicin or vincristine. This underlines the advantage of combining drugs mainly targeting the proliferative malignant cells with stroma-targeted drugs.

Previous studies have described neuroblastoma to possess an immunosuppressive microenvironment. PGE_2 is known to favor tumor-promoting M2 polarization and differentiation of monocytes. In paper II we observed a higher infiltration of M2 macrophages in high-risk tumors and in paper III we could induce a shift in M1/M2 macrophage polarization supporting anti-tumor immunity by mPGES-1 inhibition. To evaluate if MCTS could be used as a model to study inflammatory activity in neuroblastoma and if inhibition of PGE_2 production could potentiate an induced anti-tumor immunity response, peripheral blood mononucleated cells (PBMCs) were added to the MCTS. By inducing PBMCs with INFγ and LPS, we aimed to simulate an anti-tumor Th1 response and classical M1 activation. Analysis of supernatants from MCTS grown with activated PBMC revealed an M1 polarization cytokine/chemokine profile compared to non-activated PBMCs. IHC analysis showed expression of the macrophage marker CD68 in MCTS with both activated and non-activated PBMCs. The M2 polarization marker CD163 however, was exclusively expressed in MCTS grown with non-activated PBMCs. Addition of activated

PBMCs significantly reduced the growth of MCTS and mPGES-1 inhibition significantly enhanced this growth reduction.

Taken together, establishment of a three dimensional tumor model containing both neoplastic and stromal cells more accurately reflect the complex cell interaction and heterogeneity of neuroblastoma pathobiology than conventional two-dimensional monocultures. This model provides a relevant *in vitro* tool for screening of stroma targeting drugs in combination with established cancer therapies.

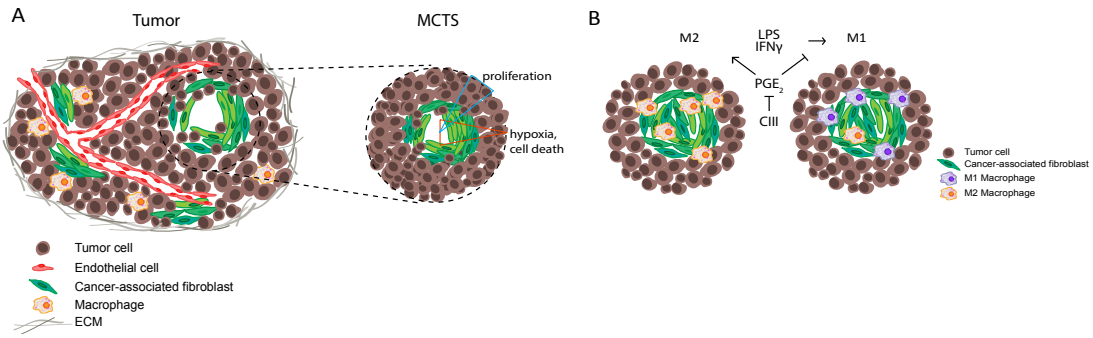

Figure 9. (A) Illustration of the local tumor milieu that is simulated in a multicellular tumor sphere. (B) Model of immune activation. Simulation of Th1 immune response with IFNγ and LPS resulting in induction of the anti-tumoral M1 polarization of monocytes. CIII treatment inhibits the immunosuppressive properties of PGE_2 known to promote M2 polarization of macrophages.

5 CONCLUSION AND FURTHER PERSPECTIVES

During the 21st century there have been an impressive increase in survival of children with neuroblastoma due to intensified therapy with combination of chemotherapy, surgery and radiotherapy. However, despite this progress the prognosis for children within the high-risk group are still poor and these children often suffers from severe side effects. In addition, in the last decades the impact of the conventional therapies on survival seems to have reached a plateau. This calls for the need of improved biological understanding and novel targeted therapies that improve survival with fewer side effects. In this thesis we provide a deeper biological understanding of the complex tumor promoting cell interactions conducted via PGE_2 within the neuroblastoma microenvironment.

In paper I we demonstrate that neuroblastoma cells abundantly express all EP receptors and that PGE_2 results in activation of downstream cancer survival signaling cascades. This suggests that selective inhibition of PGE_2 signaling by receptor antagonists could provide a therapeutic approach. However, PGE_2 signaling is complex, conducted via several receptors expressed by several cells within the tumor and are known to be trans-activated via other receptors. Consequently, selective EP receptor targeting is difficult. Even if one of the EP

receptors will be proven to play a more important role in cancer growth, single receptor targeting might not be sufficient to improve patient outcome.

In paper II we reveal an active COX/mPGES-1/PGE_2 pathway in 11q-deletd neuroblastomas with high expression o mPGES-1 and low 15-PGDH expression.
In addition, we demonstrate high-risk neuroblastoma tumors to present an immunosuppressive microenvironment and that cancer-associated fibroblasts play a role in the complex tumor-stroma interactions and might promote anti-cancer inflammation via PGE_2 production. These results imply mPGES-1 inhibition as a therapeutic approach in high-risk neuroblastoma.

In paper III we show that pharmacological mPGES-1 inhibition modulates the microenvironment and significantly inhibits tumor growth, shown both in 11-q deleted neuroblastoma xenografts and in an aggressive MYCN driven transgenic model. We therefore conclude that a seemingly non-toxic treatment targeting non-malignant cells in the inflammatory tumor-promoting microenvironment may constitute a novel clinical therapeutic approach for children with high-risk neuroblastoma.

In this thesis the importance of stromal cells within the neuroblastoma microenvironment has become evident. There is accumulating evidence of a strong link between the tumor microenvironment on drug response and disease progression in neuroblastoma. In paper IV establishment of a model aiming to mimic the microenvironment of neuroblastoma enables pharmacological mPGES-1 inhibition in combination with conventional cancer therapies *in vitro,* targeting both malignant cells and stromal cells. mPGES-1 inhibition enhances the cytotoxic effect of established chemotherapeutic drugs.
Taken together we show that pharmacological inhibition of mPGES-1 in the neuroblastoma microenvironment provides a promising therapeutic alternative to recapitulate the potent effect of COX inhibition on tumor growth without the severe side effects. Since no signs of toxicity were observed in mice treated with an mPGES-1 inhibitor over a longer period of time we propose mPGES-1 inhibition for future clinical applications as an adjuvant treatment or long-term maintenance treatment for neuroblastoma high-risk patients.

A wide verity of potential therapeutic targets has been proposed in the post-genomic era. However, the therapeutic effect of novel drugs in the cancer field still relies on tumor cell lines cultured in two-dimensions. As a result the approval rate of targeted therapies is only 5-7%. There is an urgent need to improve the preclinical cancer models so that they mimic as close as possible the malignat cancers seen in patients. In paper IV we show that three dimensional multicellular models provides a tool that more closely recapitulates *in vivo* features of neuroblastoma opening up for further exploration of the complex cellular interactions in the microenvironment and screening of drugs targeting tumor-stroma interactions *in vitro*. Three-dimensional in vitro models of neuroblastoma hopefully will improve the selection of therapeutic drugs for both animal testing and clinical trial.

Personalized medicine is an emerging field in neuroblastoma research and there is an increased interest in using primary cancer cells and tissue for drug screening. Establishment of patient derived xenografts as preclinical models for neuroblastoma have been successful with retained features of patient tumors, especially patient derived orthotopic xenografts seems to be a promising model since they give rise to metastasis in contrast to cell-line derived xenografts (Braekeveldt and Bexell, 2017; Braekeveldt et al., 2016). However, these models lack a normal immune system impeding immune therapy trials, another successful and growing field in pediatric cancers (Kopp and Katsanis, 2016). This has led to the development of humanized mouse models where mice are engrafted with human hematopoietic stem cells resulting in a functional human immune system. However these mouse models are costly and in addition the access to patient material is limited.

In conclusion, the complex interaction within the neuroblastoma microenvironment is an important filed of investigation in the search of novel therapeutic targets that can improve survival and reduce severe side effects. In order to do so we need reliable preclinical models.

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